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It is generally agreed that the Wood thrush, Hylocichla mustelina, is very closely related to four other North American spotted-breasted thrushes and, as such, all five should be placed in the same genus. However, other authoritative sources place these four other species in the genus Catharus, and leave H. mustelina as the only species in the genus Hylocichla.

A proposal has been made to place H. mustelina in the genus Turdus based on behavioral and ecological information. Evidence from serological tests have also supported this proposal.

An electrophoretic investigation of blood proteins from the involved species was started to help clarify this taxonomic problem. The electrophoretic patterns obtained from lactic dehydrogenase and hemoglobin in polyacrylamide gels were almost identical for H. mustelina and the other four woodland thrush species. The patterns of Turdus species were quite similar to one another, but distinct from patterns of either Catharus or Hylocichla specimens.

Based on the results of this study, the Wood thrush should not be included in the genus Turdus.

AN ELECTROPHORETIC STUDY OF BLOOD PROTEINS TO DETERMINE THE GENERIC POSITION OF *HYLOCICHLA MUSTELINA* (AVES: TURDIDAE)

by

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INTRODUCTION

Five species are presently included in the genus Hylocichla of the family Turdidae according to the A. O. U. Check-List of North American Birds (1961). All five are small, brown, spotted-breasted thrushes. They are often referred to as the North American woodland thrushes because of their preference for the forest understory. Their summer breeding area generally includes central and northern North America, while the winter area covers the southern-most part of the United States and Central America (Bent, 1964). Their food consists primarily of insects and fruit. All are considered fine singers.

All five species were described and named by various ornithologists between 1788 and 1848. They were initially placed in the genus Turdus because of their superficial resemblance to the European thrushes.

The genus Hylocichla was established by Baird in 1864 with the Wood thrush as the type species (Ridgway, 1907). Baird included no other species in the genus at that time. The four other North American woodland thrushes were later referred to this genus.

The genus Catharus was established by Bonaparte in 1851 which included several Central and South American forest thrushes (Ridgway, 1907). The species presently in

the genera Hylocichla and Turdus were considered to be closely related when the North American woodland thrushes were being described. However, Ridgway was one of the first to point out the close relationship between Catharus and Hylocichla (Ripley, 1952). He based his conclusion on his own observations of their structure and habits. Ridgway (1907) also discarded the idea of a close relationship between the species of Hylocichla and European Turdus species. He made a point of noting that a European thrush, Turdus musicus, should not be referred to the genus Hylocichla, as some authors had suggested, based on his own morphological comparisons.

Dorst (1950) tried to revive the idea of a close relationship between the genus Turdus and the genus Hylocichla. He included Hylocichla in Turdus based primarily on the superficial resemblance between the European Song thrush and the Wood thrush.

Ripley made a revision of the family Turdidae in 1952. He discarded the idea of Dorst based on his familiarity with both involved species in life. "The resemblance seems to be more one of parallelism in external appearance only." He went on to describe morphological and habit differences between the two species. Ripley agreed with Ridgway (1907) that the members of Catharus and Hylocichla are closely related. He noted that there are no differences in size or habits between the two genera, except Catharus species are

not famous for their fine song. He considered the members of both genera to be so close that they should be merged into one genus. "It seems wiser to merge both groups into one genus of which Catharus is the oldest name."

Dilger (1956) made a study of the relationships of the thrush genera Catharus and Hylocichla. He agreed with Ridgway (1907) and Ripley (1952) that the two genera are closely related. His conclusions were based on his own studies of morphology, ecology, behavior, and migration. "A misfit in the rather homogeneous Catharus-Hylocichla assemblage is the Wood Thrush, H. mustelina." He considered the Wood thrush to be very closely related to members of the genus Turdus, especially to Turdus musicus, the European Song thrush. He did not make any direct comparison between T. musicus and the Wood thrush; but he used Turdus migratorius, the American Robin, as a direct comparison. Between these two species, he noted similarities in nest building and hostile behavior. He left the Wood thrush in the genus Hylocichla, and placed the other four species in the genus Catharus. He indicated that further investigation would probably place the Wood thrush in the genus Turdus.

Bourns (1967) made a series of serological tests on muscle and blood proteins among the five species of Hylocichla and the American Robin. In this study, the Wood thrush showed a closer relationship to the Robin than to the remaining four species.

One European thrush species, Turdus musicus, is a source of confusion in both scientific and common name in literature about thrushes. Linnaeus described in Systema Naturae (1758) the species, Turdus iliacus, the Redwing (also Red-Winged) thrush (Ridgway, 1907). He described in Systema Naturae (1766) and Fauna Svecica (1761) the species, Turdus musicus, the Song thrush (Peters, 1964). In later classifications, these two birds have been categorized as subspecies which has lead to the confusion. Ripley (1952) lists Turdus musicus coburni for T. iliacus and Turdus musicus musicus for T. musicus. Peters (1964) lists Turdus iliacus coburni for T. iliacus and Turdus iliacus iliacus for T. musicus. A footnote in Peters (1964) states that the name Turdus musicus has been placed on the Official Index of Rejected and Invalid Names in Zoology, in 1959, by the International Commission of Nomenclature. However, T. musicus is still being used in literature. The A. O. U. Check-List of North American Birds (1961) still uses this name. T. iliacus coburni is now called the Iceland Red-Winged thrush (Bent, 1964). T. iliacus iliacus is now called the Red-Winged thrush (Peterson, Mountfort, and Hollom, 1966). Turdus philomelos (formally Turdus erecitorum) is now called the Song thrush (Peterson, Mountfort, and Hollom, 1966). Other species have been called either T. musicus or the Song thrush in the older literature; therefore, much care must be exercised in interpreting the exact species or subspecies.

The main objective would seem to be at this point to discover the "real" relationship of the Wood thrush to the other Hylocichla species and to Turdus species. If H. mustelina should be found to be sufficiently different from the other woodland thrushes and sufficiently close to Turdus species, then two problems arise; the generic position of the Wood thrush, and the generic position of the other woodland thrushes. The Wood thrush could be placed in the genus Turdus and the genus Hylocichla dropped from classification systems, since H. mustelina was the type for this genus; or the Wood thrush could be retained as the only species in the genus Hylocichla. The other four species could be merged into Catharus, if enough evidence was available to warrant this; or they could be placed into a new genus.

If the Wood thrush is sufficiently like the other four species, but different from Turdus species; then another problem arises. The woodland thrushes could be left in the genus Hylocichla, or they could be merged with the members of the genus Catharus.

Electrophoresis of proteins is one of the more recent biochemical methods being applied to taxonomic problems. Taxonomy has in the past relied on morphological, ecological, and behavioral characteristics. Protein comparison is now well founded as a reliable method for discovering phylogenetic relationships as discussed by Sibley (1962, 1967), Dessauer (1966), and Dessauer and Fox (1964).

There have been many comparative studies of blood proteins of various animals at different taxonomic levels. A great deal of this work has been reviewed by Engle and Woods (1960) and Sibley and Hendrickson (1970). However, very little work has been reported on avian species at the generic level. Baker and Hanson (1966) reported small differences which separated two closely related genera of geese. More work has been done on reptiles and amphibians at this level. Results published by Zweig and Crenshaw (1957), Voris (1967), Fox et al. (1961), Hebard (1964), and Coates (1967) have shown specific and generic differences among the species examined.

An electrophoretic survey of the blood proteins of the involved species was undertaken to help clarify the generic position of the Wood thrush.

MATERIALS AND METHODS

The majority of the specimens used were caught in the area of Greensboro, North Carolina, using Japanese mist nets. The specimens were caught in the fall of 1968, and in the spring and summer of 1969. The species and the numbers collected consist of the following: six Olive-backed thrushes, Hylocichla ustulata; fifteen Wood thrushes, Hylocichla mustelina; ten Hermit thrushes, Hylocichla guttata; eight Veerys, Hylocichla fuscescens; six Gray-cheeked thrushes, Hylocichla minima; and four Robins, Turdus migratorius.

In addition to the captured specimens, a group of frozen plasma samples were obtained from Yale University through the courtesy of Dr. C. G. Sibley. This group contained one sample of each of the following European species: the Fieldfare, Turdus pilaris; the Redwing thrush, Turdus iliacus; and the Song thrush, Turdus philomelos. One sample of each of the following South American thrushes were included; the Clay-colored robin, Turdus grayi; and the Ruddy-capped Nightingale thrush, Catharus frantzii. This group also contained one sample of each of the species H. guttata and H. fuscescens.

Blood samples were collected in a 10% EDTA solution from live specimens. The plasma was separated from whole blood samples by centrifugation and frozen immediately. The remaining red blood corpuscles were washed three times in an

isotonic salt solution and lysed with distilled water. The red blood cell fragments were removed by centrifugation, and the supernatant hemoglobin solution was decanted and frozen.

Electrophoresis was carried out in a vertical gel electrophoresis cell, Model E-C470, manufactured by E-C Apparatus Corporation. Operating procedures followed the E-C Technical Bulletin 128. All runs were made in 7% polyacrylamide gels using the standard buffer, Tris-Na_2EDTA-Boric Acid pH 8.4. The samples were run anodally with the voltage at 300 volts. The temperature of the circulating water within the unit was kept in the range 2-15° C. Gels containing plasma samples were run for two hours, while hemoglobin samples were run for three hours. Twenty microliters of sample were used in each gel slot. Bromphenol blue was added to all samples to serve as a marker during electrophoretic migration. Granules of sucrose were added to hemoglobin samples to increase density in order to keep the samples in the gel slots.

Gels containing either hemoglobin or plasma samples were stained with Amido Black 10B according to E-C Technical Bulletin 128. A prestain method to stain for lipoproteins as described in E-C Bulletin 134 was attempted without success. A periodic acid-Schiff reaction was used to stain for glycoproteins according to E-C Bulletin 143. Copper detection following two methods described by Wieme (1965), a modified Alizarin Blue S stain and a rubeanic acid stain, was attempted without success. Lactic dehydrogenase isozymes were

stained for by the method described in E-C Bulletin 144. Malic dehydrogenase isozymes were sought following the method of lactic dehydrogenase isozyme staining, but substituting malic acid for sodium lactate. No malate dehydrogenase activity was found. A peroxidase stain was used to test for hemoglobin-haptoglobin complexes as described in E-C Bulletin 145. Staining for alkaline phosphatases followed the method described in E-C Bulletin 146. The Canalco 800 series stain was used to detect the presence of transferrins.

A standard sample was used as a reference point on all gels so that migration distance could be correlated among gels. Material from a captive Ring-billed gull, Larus delawarensis, was used as the standard.

The patterns on the gels were traced and reproduced on graph paper. The fastest portion of the pattern of the standard was used as a reference point. Reference values (R_f values) were obtained by dividing the distance of the protein from the application point by the distance of the standard from the application point and multiplying by one hundred.

RESULTS

Data was obtained from gels containing either plasma or hemoglobin samples. Since the development of the electrophoretic technique, it has become a custom to classify the major plasma proteins into five groups according to the rate at which they migrate in an electric field. Proceeding from the fastest to the slowest component in human plasma, these groups are albumen, alpha-globulin, beta-globulin, fibrinogen, and gamma-globulin (Martin, 1961). Avian plasma also contains these same basic groups (Baker and Hanson, 1966). When numbering protein bands, the fastest band is assigned the number one and so on to the slowest band.

In addition to the above, there are many other proteins, carbohydrates, hormones, lipids, amino acids, free ions, enzymes, and waste products in plasma. The level of these substances varies because of many factors such as age, sex, disease, season, etc. (Fox and Foster, 1957). The plasma pattern of a species shows a high degree of uniformity with some variations because of the above factors (Morris and Courtice, 1955).

The characteristic and most important constituent of the red blood corpuscle is a protein known as hemoglobin. Hemoglobin is a conjugated protein composed of four heme groups and a protein called globin.

Electrophoresis of hemoglobin from the involved species resulted in two protein bands. Bush (1967) and Baker et al. (1966) also reported two bands for the avian species in their studies. Figure 1 is a population-range diagram for both hemoglobin bands drawn according to the method described by Mayr et al. (1953). For each species, the horizontal line indicates the total variation of the sample; the broad portion of the line, one standard deviation on each side of the mean; and the cross bar, the mean.

The slower of the two components appears inseparable among the six species. The data for the slow component is summarized in Table 1. The range of the means covers 4 R_f units. The faster band shows more variation. This data is summarized in Table 2. T. migratorius has the fastest moving band with a mean mobility of 85.91 R_f units. This component in the Hylocichla species is slower and has a mean mobility ranging from 56.6 to 60.8 units.

From inspection of the gels, there appears to be no important difference among the slow components for any of the six species; however, among the fast components there is a visible difference.

To test these results, appropriate statistical tests were applied. An analysis of variance test using a single criterion of classification for any number of groups with unequal replications was used to test for a significant difference among the means (Steel and Torrie, 1960, p. 112-114).

Figure 1. Population-Range Diagram for Hemoglobin Components

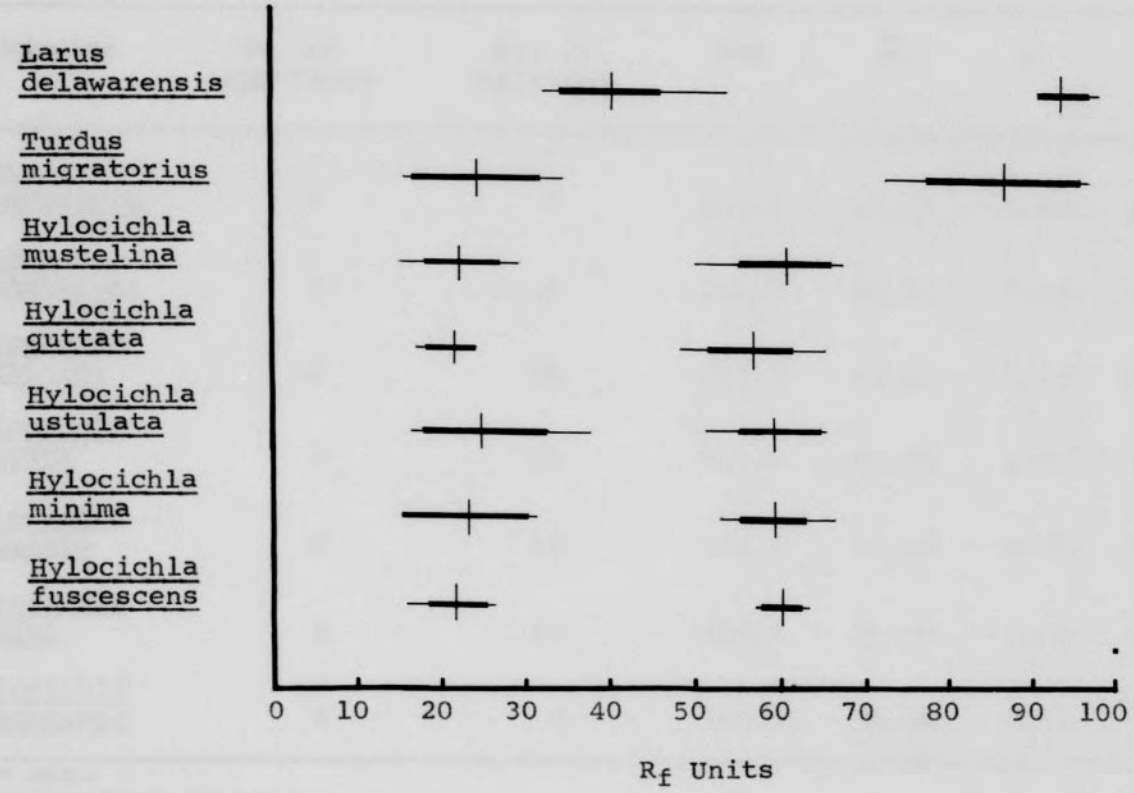

Table 1

Data for Slower Hemoglobin Component

Species	No. of Specimens	No. of Patterns	Sum	$\bar{x}$	s	$s_{\bar{x}}$
Larus delawarensis	1	9	361.0	40.11	5.80	1.93
Turdus migratorius	4	6	143.5	23.91	7.54	3.08
Hylocichla mustelina	13	18	403.0	22.39	4.22	0.99
Hylocichla guttata	9	10	212.0	21.20	2.69	0.85
Hylocichla ustulata	6	10	246.0	24.60	4.80	2.30
Hylocichla minima	5	10	227.0	22.70	4.86	2.50
Hylocichla fuscescens	8	9	193.5	21.50	3.72	1.13

$\bar{x}$ = mean
s = standard deviation
$s_{\bar{x}}$ = standard error of the mean

Table 2

Data for Faster Hemoglobin Component

Species	No. of Specimens	No. of Patterns	Sum	$\bar{x}$	s	$s_{\bar{x}}$
Larus delawarensis	1	9	833.0	92.55	2.17	0.72
Turdus migratorius	4	6	515.5	85.91	8.91	3.63
Hylocichla mustelina	13	18	1094.5	60.80	4.40	1.06
Hylocichla guttata	9	10	566.0	56.60	4.80	1.51
Hylocichla ustulata	6	10	589.0	58.90	4.86	1.53
Hylocichla minima	5	10	596.0	59.60	3.72	1.17
Hylocichla fuscescens	8	9	540.5	60.05	2.35	0.78

$\bar{x}$ = mean
s = standard deviation
$s_{\bar{x}}$ = standard error of the mean

The results of this test are shown in Table 3. Among the means of the slow components there are no significant differences, but comparison of the means of the fast components shows a significant difference. Since I suspected the difference to occur between the Hylocichla species and T. migratorius, I also did an analysis of variance on just the Hylocichla species. This test shows no significant difference among the means of these species.

Since a difference was found among the fast components, a test was applied to find between which species there was a significant difference. Tukey's w' procedure (Steel and Torrie, 1960, p. 114) is a conservative method for measuring the significance of differences between means. A difference between any two means which exceeded the w' value, 10.8 R_f units in this test, was counted as a significant difference. T. migratorius is significantly different from every species of Hylocichla, while there are no significant differences among the other species. The results are shown in Table 3. Instead of listing every difference between each two means, the species are underscored to show the differences.

Gels stained for total-plasma patterns revealed a basic pattern for each species with some variation. Within the same species, the more prominent or wider bands were usually consistent; however, some specimens of a species contained a greater or lesser number of smaller bands than other specimens. Also, a large band in one specimen occasionally appeared as a

Table 3

Comparison of Hemoglobin Means

Analysis of Variance

Comparisons	Level of significance	Critical region	Computed F	Accept H_o * Reject H_o **
Hemoglobin Slow band All species	0.05	F>2.37	F = 0.54	Accept H_o
Hemoglobin Fast band All species	0.05	F>2.37	F = 33.66	Reject H_o
Hemoglobin Fast band Hylocichla species	0.05	F>2.53	F = 1.65	Accept H_o

* H_o = Null hypothesis All means are equal.
** Accept alternative hypothesis. At least two means are not equal.

Tukey's w' Procedure

Hemoglobin - Fast Band

Species	T. migr.	H. mus.	H. gut.	H. ust.	H. min.	H. fus.
Mean	85.91	60.80	56.60	58.90	59.60	60.05
Differences *	_____	_____				

* Any two means not underscored by the same line are significantly different. Tukey's w' value equals 10.8 R_f units at a 0.01 level of significance.

number of smaller bands in another specimen. Since numbering or identification of exact bands was not possible for these specimens, comparisons between species could not be made. Staining for specific constituents of plasma was carried out more successfully.

Lactic dehydrogenase staining revealed five distinct bands or isozymes for each of the involved species. Lactic dehydrogenase can occur in five possible forms or isozymes in the organs of most vertebrates (Vesell and Brody, 1964). In previous studies, a varying number of isozymes have been reported for other avian species.

The slowest or fifth band ranged from 4 to 11 Rf units from the application point in the Hylocichla species, while this band ranged from 3 to 5 units in the species of Turdus. The fourth band in the Hylocichla species ranged from 12 to 20 units and from 8 to 10 units in the Turdus species. The third band had a range from 19 to 30 in the Hylocichla species and from 12 to 15 in the Turdus species. The range for the second band was 25 to 39 for Hylocichla and 16 to 22 for Turdus. The first band ranged from 32 to 46 for Hylocichla and from 20 to 28 for Turdus. Only the fifth band showed any overlap in range for the two groups. The distance from the application point of each band seemed to be determined by the migration of the fastest band; therefore, only the fastest band was used in statistical comparisons.

The patterns of the Hylocichla species were inseparable

from each other, but they were quite different from the patterns of the Turdus species. The one specimen of Catharus had a faint pattern, but it resembled the patterns of the Hylocichla species. The data from the Catharus specimen was included realizing the limitations of examining only one specimen of a species. Since only one specimen of each of the European and South American thrushes was available, the data from these species was combined with the data from the specimens of T. migratorius.

A population-range diagram for the fastest band of each species is shown in Figure 2. The data for the fastest band is summarized in Table 4.

The same analysis of variance procedure was used to test for a significant difference among the means. The results are shown in Table 5. There is a significant difference when all of the species are compared. I also compared just the Hylocichla and Catharus species, and the means appear as a homogeneous group. A comparison of just the Hylocichla species also results in no significant difference.

Tukey's w' test was used to verify between which means there were significant differences. A difference of more than the w' value, 6.56 units, between two means was counted as being significant. The results from this test are also shown in Table 5. The combined group of Turdus species are significantly different from every species of Hylocichla.

Gels stained for glycoproteins gave various results.

Figure 2. Population-Range Diagram for Fastest LDH Components

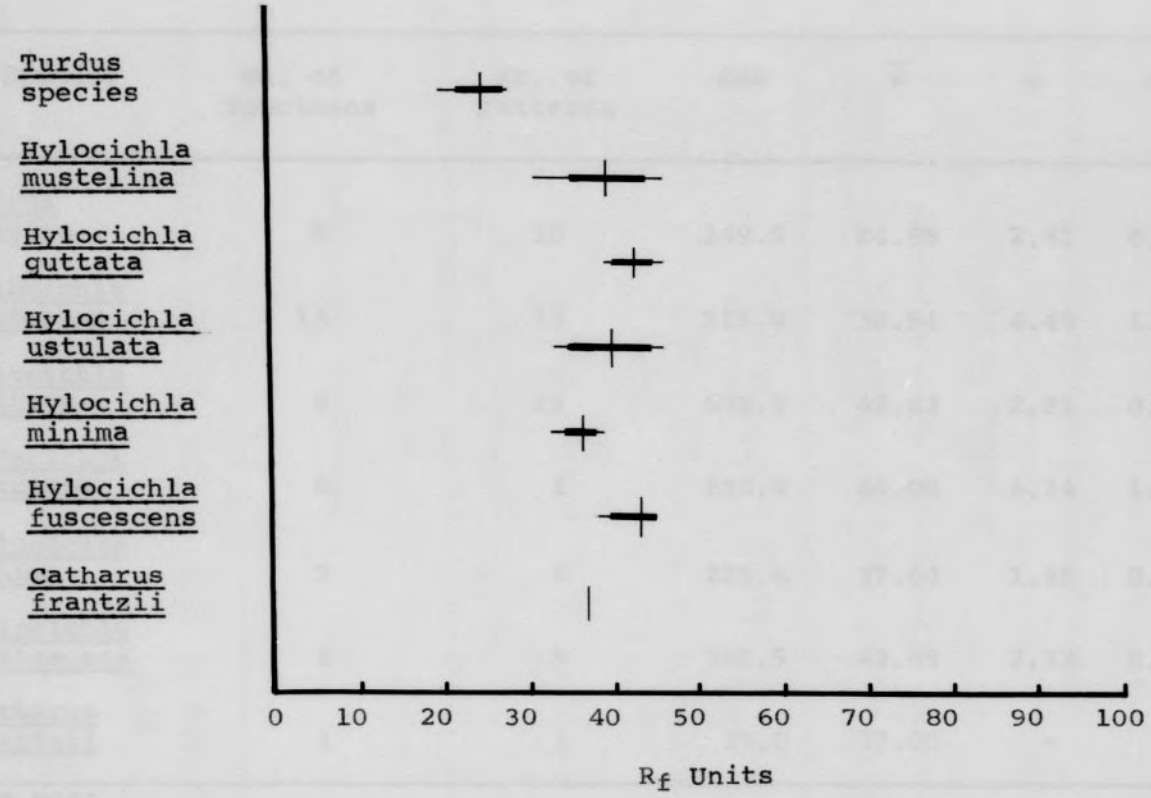

Table 4

Data for Fastest LDH Component

Species	No. of Specimens	No. of Patterns	Sum	$\bar{x}$	s	$s_{\bar{x}}$
Turdus species	8	10	249.5	24.95	2.81	0.88
Hylocichla mustelina	13	13	515.0	39.61	4.49	1.24
Hylocichla guttata	9	15	636.5	42.43	2.21	0.57
Hylocichla ustulata	6	6	240.0	40.00	4.74	1.93
Hylocichla minima	5	6	225.6	37.60	1.95	0.80
Hylocichla fuscescens	8	9	387.5	43.05	2.33	0.77
Catharus frantzii	1	1	37.0	37.00	-	-

x = mean
s = standard deviation
$s_{\bar{x}}$ = standard error of the mean

Table 5

Comparison of Fastest LDH Component Means

Analysis of Variance

Comparisons	Level of significance	Critical region	Computed F	Accept H_o * Reject H_o **
All species	0.05	F 2.25	F = 38.64	Reject H_o
	0.01	F 3.12		Reject H_o
Hylocichla and *Catharus* species	0.05	F 2.45	F = 3.38	Reject H_o
	0.01	F 3.51		Accept H_o
Hylocichla species	0.05	F 2.61	F = 3.78	Reject H_o
	0.01	F 3.83		Accept H_o

* H_o = Null hypothesis All means are equal.
** Accept alternative hypothesis. At least two means are not equal.

Tukey's w' Procedure

Species	*T. spe.*	*H. mus.*	*H. gut.*	*H. ust.*	*H. min.*	*H. fus.*	*C. fr.*
Mean	24.95	39.61	42.43	40.00	37.60	43.05	37.00
Differences *	_____	_____					

* Any two means not underscored by the same line are significantly different. Tukey's w' value equals 6.56 R_f units at a 0.01 level of significance.

Some specimens from the same species showed distinct bands, while others showed a faint stain from the application point to the end of the migration path. In some gels the bands were so faint that the bands could not be accurately reproduced. No basic pattern for glycoproteins could be established; therefore, no comparisons between species was possible.

Hemoglobin-binding haptoglobin complexes are a part of the group of proteins known as alpha-globulins (Phelps and Putnam, 1960). Gels stained for haptoglobin gave very irregular results. Some specimens showed a faint smear all along the path of protein migration. A few samples from new material gave an intense reaction, but the bands from this material were varied. No comparisons could be made from these gels.

Gels stained for alkaline phosphatases also gave inconclusive results. The only species which consistently stained for this enzyme was the Wood thrush. Older specimens of this species produced faint bands, while spring specimens produced intense bands.

Gels stained for transferrins, an iron-binding protein, gave fairly consistent but variable results. Different bands showed up in specimens from the same species; therefore, no comparisons could be made from this data.

DISCUSSION

A great deal of time was spent trying to detect the presence of copper, malic dehydrogenase isozymes, and lipoproteins; however, none of these detection procedures gave any results. Results from staining for glycoproteins, alkaline phosphatases, haptoglobins, and transferrins were varied or inconsistent; therefore, reliable conclusions could not be made from this data. Some of the above procedures would probably give better results if more time and fresh material were available.

On the gels containing hemoglobin samples, a difference was obvious between the species of Hylocichla and T. migratorius. The pattern of T. migratorius could be readily picked out from among the other species. Statistical tests showed that this observed difference was indeed a statistically significant difference.

A difference was also apparent between the species of Turdus and the species of Hylocichla and Catharus on the gels stained for lactic dehydrogenase isozymes. This difference was also shown to be significant by statistics.

Only one sample from one species of Catharus was available for this study. The pattern of this sample very closely resembled the patterns of the Hylocichla species on the lactic dehydrogenase gels. Morphological and other comparisons

made by Ridgway (1907), Ripley (1952), and Dilger (1956) argue for a close relationship between these two genera; therefore, the data for this sample was included.

Also, only one sample of each of the European and South American thrush species was available. The data from these species was combined with the data from the specimens of T. migratorius to be used as a group in comparisons. This group appeared to be a fairly homogeneous assemblage judging from the similarity of their isozyme patterns. In addition, no one has seriously suggested separating these species into two or more genera which also argues for their treatment as a homogeneous group.

The protein mobilities of the five species of woodland thrushes showed a great similarity to each other, but they were distinct from the mobilities of the species of Turdus. This evidence indicates that the Wood thrush and the other woodland species are closely related and should be united in the same genus. At the same time, a close relationship is not indicated between the species of Turdus and the woodland species; and these two groups should be placed in distinct genera. The specimen of Catharus also showed a close similarity to the woodland thrushes, but this in not enough evidence to determine if the two genera, Hylocichla and Catharus, should be merged into one. For the present, I believe it is best to classify the five species of woodland thrushes in the genus Hylocichla.

SUMMARY

After reviewing the literature on thrushes, it was apparent that the generic position of the North American woodland thrushes, especially the Wood thrush, is still an area of taxonomic disagreement. All five of these species are presently placed in either the genus Hylocichla or in the genus Catharus in classification systems. A proposal has been made to include the Wood thrush in the genus Turdus based on evidence that a close relationship exists between the Wood thrush and Turdus species.

An electrophoretic survey of blood proteins from the available species was carried out in order to obtain additional data on this problem. Only two staining procedures gave consistent enough results to be used in comparisons. The data obtained from hemoglobin and lactic dehydrogenase staining was used for the basis of the conclusions in this study.

The five species of woodland thrushes showed a very close relationship to each other, but none of these species showed a close relationship to any of the species of Turdus used. The pattern of the one species of Catharus examined resembled those of the woodland species; however, no sound conclusions could be based on this limited data.

Based on this evidence, the woodland thrushes should be

classified together for the present in the genus Hylocichla. More evidence is needed to verify the relationship between the genera Catharus and Hylocichla; however, it is clear that the Wood thrush should not be included in the genus Turdus.

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